PREIMPLANTATION DEVELOPMENT *IN VIVO* AND *IN VITRO* IN BANK VOLES, *CLETHRIONOMYS GLAREOLUS*, TREATED WITH PMSG AND HCG

EWA T. MYSTKOWSKA

*Laboratory of Experimental Embryology, Institute of Obstetrics and Gynaecology,
Medical Academy, Warsaw, Poland*

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Summary. Ovulation can be induced in the bank vole by PMSG and HCG and takes place $9\frac{1}{2}$ to $11\frac{1}{2}$ hr after administration of HCG. The number of eggs obtained varied from 1 to 30 (average 9.3). The highest ovulation rates were observed when PMSG and HCG were administered 41 to $43\frac{1}{2}$ hr apart. The embryos attained the blastocyst stage 96 hr after HCG injection. Bank vole eggs can be cultured in chemically defined media beginning with the eight-cell stage; only 25% of four-cell eggs developed to blastocysts *in vitro*.

INTRODUCTION

Although the bank vole is bred in several laboratories (see Festing, 1971), very little is known about the reproduction and embryonic development of this species (Brambell & Rowlands, 1936; Clarke, Clulow & Grieg, 1970).

The aim of the present studies was threefold: first, to investigate the ability of gonadotrophins (PMSG and HCG) to cause ovulation; second, to develop a method of culturing cleaving eggs; and third, to collect data on the rate of preimplantation development *in vivo* and *in vitro*.

MATERIALS AND METHODS

The animals used in the present study were obtained from the Mammals Research Institute in Białowieża. For the duration of the experiments (2 to 5 months), females were kept in groups of five to eight in cages measuring $30 \times 20 \times 14$ cm, and males were kept singly. The lighting regimen was 13 to 14 hr light/24 hr (lights from 07.00 hours) and the room temperature varied from 17 to 20°C. Food was provided in excess once daily and consisted of seeds, red beet, carrot and specially granulated food for rabbits.

Induction of ovulation with PMSG and HCG

Females were given an intraperitoneal injection of 5 i.u. PMSG (Gestyl: Organon) followed by intraperitoneal administration of 5 i.u. HCG (Biogonadyl: Biomed) 35 to 50 hr later. The PMSG injection was given at various times between 06.00 and 23.00 hours. Immediately after the second injection,

the females were caged with males and inspected for the presence of a vaginal plug $4\frac{1}{2}$ to 12 hr later. The animals were killed $8\frac{1}{2}$ to 96 hr after the injection of HCG and the embryos were flushed from the oviduct or the uterus with Ringer solution supplemented with bovine plasma albumin. Females that did not mate were not given another hormonal treatment for at least 14 days. Twelve females, which were not placed with males, were killed at 30-min intervals from $8\frac{1}{2}$ to 14 hr after the administration of HCG.

Development in vivo

The ages of embryos flushed from the oviducts and uteri were expressed as the time from the HCG injection (Table 2), but the real age of embryos could be determined from the time of ovulation, i.e. $9\frac{1}{2}$ to $11\frac{1}{2}$ hr after the HCG injection. The number of cells in embryos aged 64 hr or more was determined on air-dried preparations made by the technique of Tarkowski (1966).

Development in vitro

The embryos were cultured in Whitten's medium (Whitten, 1971) and in the medium devised by J. G. Mulnard (personal communication). The composition of Mulnard's medium is as follows (in mg/100 ml): NaCl, 757; KCl, 35; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 29; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 16; calcium lactate. $5\text{H}_2\text{O}$, 71; glucose, 83; sodium pyruvate, 3; phenol red, 2; NaHCO_3 , 100; bovine plasma albumin, fraction V₁, 300; streptomycin, 5; penicillin, 7.5. In some experiments, this medium was slightly modified and contained 140 mg NaHCO_3 and 727 mg NaCl/100 ml. The eggs were cultured in drops of medium under liquid paraffin (Tarkowski, 1961; Brinster, 1963). The following gas mixtures were used: exhaled air; 3% CO_2 in air; 6.2% CO_2 in air; and 3% CO_2 , 5% O_2 and 92% N_2 .

RESULTS

Induction of ovulation

The highest average number of eggs was obtained when PMSG and HCG were given 41 to $43\frac{1}{2}$ hr apart (Table 1).

Of the 236 females which were given PMSG and HCG, 122 (51.7%) mated. Among ninety-eight females which had mated and which were subsequently killed, no eggs could be found in the oviducts of eight (8.2%) and all the eggs were unfertilized in another four (4.0%). Of the 796 eggs obtained from the remaining eighty-six females, there were 664 developing eggs (an average of 7.7 embryos/female), fifty-one unfertilized eggs and eighty-one degenerated eggs. The number of eggs in individual females varied from one to thirty (Text-fig. 1).

Development beyond implantation may occur after hormonally induced ovulation and mating. Three females left until term gave birth to two, five and nine young, respectively, but only four of sixteen females killed between the 5th and 9th day of pregnancy had implanted embryos (mean = 4.2/female).

In the females treated with PMSG and HCG but not placed with a male, two killed $8\frac{1}{2}$ and 9 hr after the HCG injection had not ovulated. Two females killed after $9\frac{1}{2}$ and 10 hr had seven and four eggs in only one oviduct, and three

Table 1. The relationship between the number of eggs ovulated in voles and the interval between PMSG and HCG

	Interval between injections of PMSG and HCG (hr)					
	35 to 37½	38 to 40½	41 to 43½	44 to 46½	47 to 49½	50 to 52½
No. of females	5	6	31	22	20	2
Total no. of ova	44	41	291	164	114	9
Mean no. of ova/female	8.8	6.8	9.4	7.5	5.7	4.5

TEXT-FIG. 1. The number of ovulated eggs (open columns) and developing eggs (hatched columns) in bank voles after treatment with PMSG and HCG.**Table 2.** Rate of development of bank vole eggs *in vivo*

Time after HCG injection (hr)	Total no. of eggs	No. of blastomeres									Blasto- cysts
		1	2	3	4	4 to 8	8	8 to 16	16	> 16	
24 to 26	41	41	.	.	.	.	.	.	.	.	.
28 to 30	29	1	28	.	.	.	.	.	.	.	.
42 to 46	23	.	20	1	2	.	.	.	.	.	.
49 to 49.3	6	.	1	2	3	.	.	.	.	.	.
50 to 51.3	73	.	1	4	62	.	.	.	.	.	.
52 to 54.3	28	.	.	1	2	3	22	.	.	.	.
55 to 58	46	.	.	.	17	4	25	.	.	.	.
60 to 61	26	.	.	1	9	3	13	.	.	.	.
64 to 66	15	.	.	.	.	1	7	7	.	.	.
67 to 68	13	.	1	.	.	.	3	1	8	.	.
69 to 70	27	.	.	.	.	1	.	10	10	6	.
71 to 72	26	.	.	.	.	.	.	4	14	8	.
96	14	.	.	.	.	.	.	.	.	.	14

females killed between 10½ and 11½ hr had seventeen, fifteen and nineteen eggs. Two of five females killed between 11½ and 14 hr had twenty and twelve eggs; no eggs could be found in the oviducts of the other three animals. It seems, therefore, that ovulation starts about 9½ hr and is completed by 11½ hr after the injection of HCG.

Development in vivo

As shown in Table 2, embryos were in the one-cell stage up to 26 hr after HCG treatment. The first cleavage occurred between the 26th and 28th hr. The second cleavage took place between the 45th and 50th hr. Some embryos reached the eight-cell stage between 58 and 61 hr but after 64 hr the rate of development of individual embryos varied considerably, e.g. the embryos recovered from one female 69 hr after the HCG injection consisted of seven to sixteen blastomeres. After 96 hr, blastocysts were found in the uterus and each consisted on average of 49.4 cells including 2.5 metaphase plates (mean from fourteen blastocysts).

Table 3. Culture of four- and eight-cell bank vole eggs *in vitro*

Medium	Gas mixture	Stage of embryo	Developed blastocysts		Mean no. of cells
			No.	%	
Whitten	3% CO ₂ , 5% O ₂ , 92% N ₂	4-cell	11/43	25.6	39.1
		8-cell	12/29	41.3	56.7
Whitten	6.2% CO ₂ in air	4-cell	6/25	24.0	46.0
		8-cell	1/1	100.0	63.0
Whitten	3% CO ₂ in air	8-cell	10/18	55.5	58.3
Whitten	Exhaled air	8-cell	13/18	72.2	34.9
Mulnard	Exhaled air	8-cell	5/13	38.4	49.0
1 g NaHCO ₃ /l					
Mulnard	Exhaled air	4-cell	2/22	9.0	28.5
1.4 g NaHCO ₃ /l		8-cell	67/77	87.0	40.8

Development in vitro

The development of four- and eight-cell stages in various gas mixtures is shown in Table 3; one- and two-cell eggs underwent only one cleavage and were arrested in development. The best culture conditions for four-cell embryos were Whitten's medium and a gas mixture of 3% CO₂, 5% O₂ and 92% N₂; the blastocyst stage was reached about 110 to 120 hr after the HCG injection. The eight-cell eggs developed to blastocysts in both media and under all the gas mixtures used, but the highest percentage of blastocysts was obtained in Mulnard's medium containing 1.4 g NaHCO₃/litre and gassed with exhaled air. The blastocyst stage was reached 98 to 110 hr after HCG injection, i.e. after 30 to 48 hr of culture. Blastocysts developed *in vitro* had a slightly smaller cell number than those recovered from the uterus 96 hr after HCG (40.0 cells compared to 49.4).

DISCUSSION

In the bank vole, ovulation can be induced by gonadotrophins (PMSG and

HCG) and takes place $9\frac{1}{2}$ to $11\frac{1}{2}$ hr after the administration of HCG, i.e. beginning and ending earlier than it would in a mouse. According to Edwards & Gates (1959) and Marston & Chang (1964), ovulation in the mouse begins 11 to 12 hr after the administration of HCG and is completed by the 14th hr, although there is some variation according to strain (Edwards & Gates, 1959). The bank vole colony used in this study was genetically diversified and this might have been expected to cause greater variability in the time of ovulation.

The number of ovulations following hormonal treatment is larger than that resulting from natural ovulations under field conditions or in the laboratory. According to Brambell & Rowlands (1936), a female bank vole under field conditions produces a mean of 4.4 ova (1 to 12) at ovulation. Under laboratory conditions, the average litter size is 3.6 (Buchalczyk, 1970). Six females which ovulated spontaneously and were killed on the 4th day of pregnancy had an average of 3.6 eggs (E. Mystkowska, unpublished). The fact that over 50% of females ovulate 5 or more eggs (7.6 on average) after hormonal treatment indicates that superovulation can be obtained in voles, although not as easily as in mice in which an average of 24.6 (Fowler & Edwards, 1957) or 21.4 (McLaren, 1962) eggs are produced after hormonal treatment.

According to Clarke *et al.* (1970), ovulation in the bank vole under laboratory conditions is evoked by mating and occurs 6 to 14 hr after copulation. In the present experiments, it was not possible to exclude mating as an additional stimulus to cause ovulation; three of twelve females isolated from males and eight out of ninety-eight females kept with a male had no eggs in the oviducts after being treated with gonadotrophins.

The present study has shown that although development beyond implantation may occur after hormonally induced ovulation and mating, it takes place relatively rarely (four out of sixteen females). It is clear, moreover, that hormonally induced ovulation and mating do not automatically lead to pregnancy, since there were a few voles in which no eggs or only unfertilized eggs were found (twelve out of ninety-eight).

The rate of development of bank vole eggs *in vivo* was similar to that of mouse eggs.

The media in which the bank vole eggs were cultured were those suitable for development of mouse eggs. Although most of the eight-cell vole eggs developed to the blastocyst stage, only 25% of four-cell stages did so, probably because they were harvested just before the third cleavage. One- and two-cell eggs cleaved only once. A similar phenomenon was observed while working on the conditions under which mouse eggs can be cultured *in vitro*. It is known that the critical stage for development of one-cell mouse eggs *in vitro* is the period between the first and second cleavage, and that mouse eggs require specific energy sources at different stages of development and exactly defined pH and osmolarity conditions (Whitten, 1957; Whitten & Biggers, 1968). The one-cell eggs develop only when pyruvate is added to the culture medium while the two-cell stage requires lactate. In the present study, the culture media contained both pyruvate and lactate but they did not support development of one- and two-cell bank vole eggs. The rôles of other factors such as pH and osmolarity have still to be investigated.

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